NOTE ON THE NATURE OF OPSONIC IMMUNITY.*

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STIMULATED by the work of Sir Almroth E. Wright, various observers have recently investigated the question of phagocytosis in connection with the theory of opsonic immunity, and have obtained the following rather generally accepted results:

1. Active phagocytosis, *in vitro*, of many of the common forms of bacteria is dependent upon the action of blood serum or similar body fluids, and does not take place when these fluids are replaced by physiological salt solution.

2. Almost no phagocytosis occurs in serum which has been exposed to a temperature of 60° C. for 10 to 30 minutes.

3. Serum is deopsonized, i. e., it is inactivated, by digestion with a bacterial emulsion with subsequent removal of the bacteria by centrifugalization.

With these results as a basis, we have carried out some of the fundamental experiments in phagocytosis. As regards the relation of opsonins to immunity, it is of importance to decide, first of all, whether or not phagocytosis, as it occurs normally, is an inherent property of the leucocytes and independent of any action of the body fluids. In view of the differences sometimes found in the serum and plasma of the same individual, for example as regards the enzyme content,[1] it would be of both clinical and scientific interest to determine whether opsonins are present in the plasma and whether the breaking down of the formed elements during the clotting of the blood has any influence on phagocytosis.

Wright and Douglas[2] found the same opsonic content both in plasma, obtained by decalcifying blood, and in the corresponding serum. This method of obtaining plasma is of questionable service in deciding the rôle of the formed elements, since mere precipitation of the calcium salts does not entirely prevent the breaking down of the cells in the shed blood. To avoid this difficulty, advantage was taken of the fact that blood, removed from the body in resected

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bloodvessels, will stand for long periods without clotting.[3] It should be possible then to resect a blood vessel, precipitate the corpuscles by centrifugalization, and pipette off the supernatant plasma without bringing it in contact with a cut surface. For studying the rôle of the cellular elements, this principle was employed in the following way.

Rabbits were used, and in preparing them no anesthetic was administered, in order to avoid any possible complications arising from its anti-phagocytic action. In order that sensation might be abolished the animals were pithed between the occipital bone and the atlas. A tracheal cannula was at once inserted and artificial respiration supplied. The heart was found to be the most suitable part of the vascular system for resection as its muscular wall afforded a substantial protection to the intima during the subsequent manipulations. Plasma obtained in this manner could not be used directly for phagocytic work, since the addition of the leucocytic emulsion would furnish the only factor lacking for coagulation. The presence of fibrin after the addition of the bacterial emulsion is objectionable since the bacteria collect in its meshes and escape the action of the leucocytes, thereby giving entirely misleading results. In order to avoid this difficulty the phagocytic preparations were not made until after spontaneous coagulation had occurred, which took place after an hour's time at room temperature. Preparations were then made in the ordinary manner.

We have followed very closely the usual opsonic technique. Staphylococcus cultures were obtained from two different sources but both were of rather low virulence. The staphylococcus emulsions were always prepared from a one-day culture on agar at 37° C.

In the preparation of the leucocytic emulsion, two washings were used. One-half to one c.c. of blood was collected in about 10 c.c. of a solution composed of 1 per cent sodium citrate and 0.85 per cent sodium chloride which was contained in an ordinary centrifuge tube. After centrifugalization and without removing the supernatant fluid, approximately 0.2 c.c. of the blood-cream, on the surface of the corpuscles, was pipetted off and rewashed with about 10 c.c. of 0.85 per cent sodium chloride solution. This procedure gave emulsions rich in leucocytes and practically devoid of phagocytic power in the absence of serum. Except where otherwise stated, the leucocytes and serum were always obtained from normal human blood. For isotonic salt solution, 0.85 per cent sodium chloride was employed.

The preparations were put up in pipettes. Equal volumes were used of each of the different fluids entering into the preparation, for example: one volume of leuco-

cytic emulsion, one volume of bacterial emulsion, and one volume of serum. Unless otherwise stated, the preparations were incubated for 30 minutes at 37° C. Smears were made as advised by Wright and were stained by Hasting's modification of Jenner's stain. In counting the preparations, we have depended upon the results obtained from enumerating the bacteria in 50 consecutive polymorphonuclear-neutrophile cells. Owing to the unavoidable errors associated with the method, no emphasis has been placed on slight quantitative variations.[4] The experiments have been confined to preparations giving practically qualitative results, i. e., either active or minimal phagocytosis.

In a typical case a count of 50 leucocytes showed a total of 653 as compared with 1,067 bacteria for a preparation with serum of the same animal collected in the ordinary manner. As a control, a third preparation with salt solution gave a count of 67 bacteria per 50 leucocytes. Although this process eliminated a very high percentage of the cellular elements, a very definite diminution in phagocytosis was not obtained. When leucocytes are tested directly, *in vivo*, for example, by the injection of typhoid bacteria into the circulation,[5] active phagocytosis at once takes place. If inflammation or fibrin-formation is excluded, this can be taken as proof of the activity of the plasma, except for the possibility that some property of the leucocytes is lost in their treatment outside of the body.

Other experiments also indicate that opsonin does not arise during the breaking down of the formed elements of the blood. Neumann[6] found that extracts of leucocytes do not favor phagocytosis. I have obtained a similar result for platelets. For their preparation, the method described by Cole was employed.[7] Normal human blood was collected in a solution containing 1.5 per cent sodium citrate and 0.85 per cent sodium chloride. The corpuscles were then separated by fractional centrifugalization. The platelets readily remained in suspension, coming down principally in the later fractions. After recentrifugalizing three or four times with salt solution, they were obtained practically free from other cells. An oese of platelets was digested in salt solution at 37° C. for two hours. Upon adding this mixture to an emulsion of leucocytes and staphylococci, no more phagocytosis resulted than in the control preparation with salt solution.

Although absolute conclusions are not justified, it seems probable that active phagocytosis occurs both in plasma and in serum.*

* In a preliminary communication, Briscoe[8] expresses a different opinion. His final results have not yet been reported.

EVIDENCE OF OPSONIC ACTION.

Wright[9,2] considers that the opsonic action of the serum consists in a specific modification of the bacteria ingested. The following experiment is the one originally devised for the demonstration of opsonic action:

Three preparations are necessary.

A. A mixture of equal volumes of bacterial emulsion, of washed leucocytes, and of serum, when incubated, gives pronounced phagocytosis.

B. Another preparation is made containing a mixture of bacterial emulsion and serum. This is first digested for 15 minutes at 37° C., and then heated at 60° C. for 10 minutes or longer. Now when leucocytes are added to this, pronounced phagocytosis also occurs.

C. As a control, a third preparation is necessary. This is similar to the second, except that the mixture of serum and bacterial emulsion is heated immediately to 60° C. without the preliminary digestion. When subsequently incubated with leucocytes, only a minimal phagocytosis occurs.

In this experiment there are two points of especial importance: (1) Some phagocytosis *does* occur in the control preparation (Prep. *C*); (2) The amount of phagocytosis after digesting and heating the bacterial emulsion (Prep. *B*) is somewhat *diminished* as compared with the untreated emulsion (Prep. *A*). These differences, in the case of cocci at least, are so definite that they are practically qualitative and are well outside the limits of experimental error.

Bulloch and Atkin[10] have confirmed these observations. We have also repeated this experiment with the following results:

	Bacteria per 50 leucocytes		
	Experiments		
Mixtures	I	II	III
A) Staphylococcus emulsion + serum + washed leucocytes......	680	726	632
B) Staphylococcus emulsion + serum, digested 15 minutes at 37° C. and heated 30 minutes at 60° C. + washed leucocytes..........	529	699	490
C) Staphylococcus emulsion + serum, mixed and heated at once for 30 minutes at 60° C. + washed leucocytes.........	208	192	114

In considering the possibility of the coexistence of opsonins and stimulins it has been noted[2] that the phagocytosis in the case of the digested and heated bacteria (Prep. *B*) is slightly diminished. The constancy of the diminution in the same direction might suggest

that some stimulin action had been destroyed during the heating. There is a simpler explanation, however, for we found that the clumping of the bacteria during the heating diminished the numbers of free cocci available for phagocytosis.

Hektoen and Ruediger[11] introduced the following variation in the preceding experiment. In the second preparation, the bacterial emulsion and serum, immediately after digestion at 37° C., were separated by centrifugalization and the bacteria were then heated to 60° C. in salt solution instead of in serum. After this treatment, the bacteria were neither phagocyted when suspended in salt solution nor even in the presence of active serum. They consider that these results contradict Wright's observations and they also draw interesting conclusions from them in regard to the structure of opsonins.

In the repetition of this modification, sensitized bacteria were obtained by digesting equal volumes of serum and staphylococcus emulsion (about 0.2 to 0.3 c.c. each) at 37° C., for 30 minutes. This mixture was then diluted with about 10 c.c. normal salt solution and the bacteria were collected by centrifugalization. An oese of the bacterial precipitate was emulsified in salt solution.

	Bacteria per 50 leucocytes Experiments	
Mixtures	I	II
A) Sensitized staphylococci + washed leucocytes + salt solution......	258	476
B) Sensitized staphylococci, heated 30 minutes at 60° C. + washed leucocytes + salt solution..............................	47	91
C) Sensitized staphylococci, heated for 30 minutes at 60° C. + washed leucocytes + serum..................................	291	379

The necessary controls show that the treatment by heat in the second preparation is responsible for the loss of phagocytic action. These results agree with Hektoen and Ruediger's except that in the third preparation we were not able to demonstrate any change in the bacteria preventing the action of the leucocytes in the presence of active serum. This difference might perhaps be accounted for by the fact that I worked with staphylococci whereas their results are given for streptococci.

The interpretation of these two experiments presents some difficulties. It is not altogether clear whether or not one can have active phagocytosis of unopsonized bacteria, in a case where serum is necessary for phagocytosis. The problem is rendered perhaps a little simpler by making a slight modification in Hektoen and Rue-

diger's experiment. Since sensitized bacteria are not phagocyted after heating, a similar result might be obtained directly, using dilute serum, instead of first sensitizing the bacteria with serum which is later to be diluted by washing.

Preparations were made as follows, using a bacterial emulsion of only moderate strength:

Mixtures	Bacteria per 50 leucocytes Experiments I	II
A) Staphylococcus emulsion + serum (1–10 dilution) + washed leucocytes	221	447
B) Staphylococcus emulsion + serum (1–10 dilution digested 20 minutes at 37° C. and heated 30 minutes at 60° C. + washed leucocytes	51	122
C) Staphylococcus emulsion + serum (1–10 dilution) mixed and heated at once for 30 minutes at 60° C. + washed leucocytes	18	30

Here we have active phagocytosis although the control preparations show no evidence of any alteration in the bacteria.

Another fundamental experiment for demonstrating the rôle of the serum in phagocytosis is as follows: Leucocytes when washed by centrifugalization with normal salt solution show no evidence, as far as phagocytosis is concerned, of any reaction with or any fixation of any part of the plasma, although they have been bathed in it continuously. On the other hand, bacteria, after digestion with normal serum, are still phagocyted actively after fairly complete removal of the serum by washing.[11] This is considered as proof of opsonic action by many workers. But the experiment upon which this statement is based does show that considerable diminution in phagocytosis after washing occurs. This diminution is attributed to a dilution of the bacterial emulsion during manipulation. Sufficient controls, though, were not recorded for discriminating between a loss of opsonin by washing and a dilution of the emulsion by loss of bacteria.

As compared with leucocytes, the mechanical difficulties are somewhat greater in applying this experiment to bacteria. In order to effect thorough washing, we used a bacterial emulsion and serum in equal quantities (about 0.2 to 0.3 c.c. each). After digestion at 37° C. for 30 minutes, a sample was reserved and the mixture diluted with 11 c.c. of normal salt solution contained in an ordinary centrifuge tube. Centrifugalization for 45 to 60 minutes with an electrically driven centrifuge (2,000 revolutions per minute) was found sufficient to throw down the bacteria. During the precipitation,

the bacteria became extensively massed together and in order to expose them satisfactorily to the washing fluid, it was necessary to emulsify them thoroughly after each precipitation. At the end of each washing the emulsion was restored to its original concentration and a small sample reserved. Two preparations were made from each sample. One contained equal volumes of washed leucocytes, bacterial emulsion, and salt solution. The second was a duplicate of this except that one volume of serum was substituted for the volume of salt solution. Such controls are necessary and sufficient to locate the factors which may be responsible for any change in phagocytosis.

The experimental data are as follows:

TABLE I.
EFFECT OF WASHING BACTERIA AFTER TREATMENT WITH NORMAL SERUM.

NUMBER OF WASHINGS	STAPHYLOCOCCUS PYOGENES AUREUS		BACILLUS TUBERCULOSIS		STAPHYLOCOCCUS PYOGENES AUREUS		APPROXIMATE DILUTION
	Serum	Salt Sol.	Serum	Salt Sol.	Serum	Salt Sol.	
Without washing	1,018	738	226	192	796	517	1–1
First washing	925	339	216	165	533	275	1–30
Second washing	1,206	49	217	104	663	92	1–500
Third washing	...	...	145	37	...	...	1–15,000
Control with salt solution	119		32		11		

The figures given express the number of bacteria per 50 leucocytes. Although the preparations with salt solution need not necessarily be equal to the corresponding serum preparations, yet there should be practically no diminution with the successive washings unless some opsonin has been lost. The approximate dilutions are stated without regard to the precipitate of bacteria present and are intended merely to give some idea of the amount of washing employed. In comparing leucocytes and bacteria with regard to the amount of washing necessary to free them from serum, one must consider the theory of washing precipitates and the phenomena of adsorption.[12] The extent of surface condensation of fluids on precipitates depends partly upon the area of exposed precipitate. In proportion to their volume, then, the bacteria offer a larger surface area than the leucocytes for the retention of serum since the area of a sphere grows proportionally smaller as its volume increases. Thus the area of a leucocyte would be approximately 10 times less than the surface area

of an equal volume of staphylococci on the basis of an average diameter of 10 microns for a leucocyte and 1 micron for a staphylococcus.

The results of this experiment do not contradict any previously reported work. The bacteria at the end of the first washing correspond entirely, in their properties, to Hektoen and Ruediger's sensitized bacteria and we have used them as such with satisfactory results. The washing experiments of Neufeld and Rimpau[13] do not concern us in this connection, since they worked with immune sera. They used only one washing, however, and since they do not state the volumes of fluid used, it is not altogether certain that the bacteria were finally freed from serum. Bächer,[14] working on this problem, came to the same conclusions which Hektoen reached. A diminution in phagocytosis was obtained in some cases, but he also attributes this to a loss of bacteria from mechanical causes. Recently, Sleeswijk[15] considers that he has demonstrated opsonic action in the case of anthrax bacilli and frog serum. This same principle was employed, but since only one washing was used, it is not unlikely that sensitized bacteria were obtained, corresponding to the bacteria usually obtained at the end of the first washing. The experiment is open to further objections. Other observers,[16] in order to obtain satisfactory preparations with anthrax, have found it necessary to work with spores on account of the long chains which the bacteria form. There is even some dispute as to whether serum is necessary for the phagocytosis of anthrax bacteria.[17, 18] Sleeswijk reports a strong agglutinating action of the frog serum upon the anthrax bacilli. This must have increased the difficulty of thoroughly exposing these agglutinated clumps to the washing fluid. These clumps must also have given further difficulty in the study of their phagocytosis. Dean[19] found that when bacteria, which have been supersaturated with serum, are suspended in salt solution, the opsonin diffuses into the surrounding medium. Centanni[34] has reported that the opsonic reaction between serum and pneumococci can readily be destroyed by washing. He also makes the important statement that after several repetitions of this process, the pneumococci are no longer phagocyted even in the presence of active serum.

This experiment does not permit us to draw final conclusions.

The possibility of opsonic action is neither proven nor disproven. One might suppose, for instance, that there is a reaction between the bacteria and the serum, perhaps a chemical reaction, which is a reversible one. Then if its direction depended upon the dilution employed, any opsonic effect might readily be destroyed by washing. These results have, however, a few points of practical importance in connection with such experiments as the following:

1. Sensitization methods have been developed especially by Hektoen and they are particularly applicable in many experiments, for example, in distinguishing between anti-opsonic and anti-phagocytic substances. If no phagocytosis resulted in a preparation where the bacteria were presumably sensitized, control tests would be necessary to differentiate between an inactive fluid and an active serum which might have been completely removed by washing.

2. If serum has no stimulating effect upon leucocytes, one might expect that after sensitizing bacteria, the serum could be discarded or that if it were injected along with the bacteria it would have no protective effect upon the inoculated animal. Bordet and Gengou's[20] study of the comparison of the injection of (*a*) untreated bacteria, of (*b*) sensitized bacteria, and of (*c*) bacteria mixed with serum, showed that the sensitized and non-sensitized bacteria killed the animals into which they were injected while the animal which received the simultaneous injection of serum survived.

DEOPSONIZING EXPERIMENTS.

The technique of the deopsonizing experiments is briefly as follows: Equal volumes of a heavy bacterial emulsion and serum are incubated at 37°C. ° for 15 to 30 minutes. The mixture is then centrifugalized until the bacteria are precipitated and the supernatant fluid is free from organisms. The bacterial sediment is discarded and the serum, when tested with a fresh emulsion of bacteria in the ordinary manner, is found to have lost most of its opsonic power.

In the case of heated immune sera and the tubercle bacillus, Wright and Reid[21] consider this single procedure as proof of opsonic action. Control tests are necessary for the final interpretation of such an experiment. Whatever may be the probable explanation, at least two possibilities demand consideration: (1) either an injurious

substance may have been introduced, or (2) a substance favoring phagocytosis may have been removed.*

The first of the two possibilities suggested might have an important bearing upon specificity determinations, but as regards the question of the chemical or physical aspects of the opsonic reaction, only the second one is of especial interest. Either the opsonin may combine chemically with the bacteria, or it may be removed mechanically from the serum by the bacterial emulsion, somewhat as enzymes are carried out of solution by foreign bodies. In support of this mechanical explanation, it is found that serum is deopsonized by bacteria at 0° C.,[23] the low temperature itself not being responsible for any deleterious action on the serum. Bulloch and Atkin[10] consider that the bacteria themselves are opsonized. To test this point, we applied the original heating experiment of Wright with the following results:

Mixtures	Bacteria per 50 leucocytes Experiments I	II
A) Staphylococcus emulsion + serum, digested 15 minutes at 37° C. and heated 30 minutes at 60° C. + washed leucocytes	490	699
B) Staphylococcus emulsion + serum, digested 15 minutes at 0° C. and heated 30 minutes at 60° C. + washed leucocytes	159	60
C) Staphylococcus emulsion + serum, heated at once for 30 minutes at 60° C. + washed leucocytes	114	192

In preparation *B*, both the serum and the bacterial emulsion were cooled to 0° C. before mixing.

Thus, although we may have serum deopsonized at 0° C., it cannot be shown that the bacteria themselves are opsonized. Cowie and Chapin[24] have carried out a variety of deopsonizing experiments in working upon the amboceptor-complement structure of normal opsonins. They find different types of deopsonized serum according to whether the amboceptor or the complement is destroyed. It might be of importance to determine what type of inactivation is effected by deopsonizing with carbon particles.

As further evidence of the possibility of the mechanical removal of opsonin, Bächer,[14] and also Simon, working with Lamar and Bispham,[25] found that serum is deopsonized by foreign substances,

* In this connection Ledingham[22] finds that "Heated normal serum through which tubercle bacilli have been passed exerts a marked inhibitory action . . . . on the opsonin of fresh normal serum." He suggests a slightly different explanation, namely, the action of opsonic amboceptors with the phenomena of deviation of the complement by free receptors thrown off from the bacteria into the heated serum.

such as carmine, charcoal, and filter paper. Charcoal is known to absorb a variety of substances, such as gases, coloring matter, proteids, and also enzymes.[26] In testing the specificity of the deopsonizing action, we compared the activity of normal serum and of serum digested with carbon at 37° C. for 20 minutes with subsequent removal of the carbon by centrifugalization.

Mixtures	Bacteria per 50 leucocytes Experiments I	II
A) Washed leucocytes + staphylococcus emulsion + serum..........	591	869
B) Washed leucocytes + staphylococcus emulsion + treated serum...	167	220

EVIDENCE OF THE ACTION OF SERUM UPON LEUCOCYTES.

Turning to the possibility of an action of the serum upon the leucocytes, it is important to remember that the theory of stimulin action does *not* necessarily involve any chemical reaction.[27] Indeed one would expect the leucocytes to have come into a state of chemical equilibrium with the surrounding plasma. It is altogether possible that the serum, though necessary for the phagocytosis, reacts, neither with the leucocytes nor with the bacteria, but it may exert its influence in a catalytic manner.

In considering whether serum has any direct effect upon leucocytes, the variable factors in the problem may be reduced by substituting a chemically inert body for the bacteria. This limits one then to the effect of the serum upon the leucocytes.

In applying this principle, Wright and Douglas[2] came to the conclusion that serum probably favored the phagocytosis both of carmine and of India-ink particles. To secure an inert body in a suitable condition for phagocytosis, we used chemically pure carbon black, suspended in normal salt solution. In emulsifying it, the smaller particles were massed together upon the addition of liquid. A satisfactory separation, suitable for subsequent enumeration, was obtained by the same process usually recommended for the preparation of tubercle bacilli emulsions. The most satisfactory preparations were secured by using dilute emulsions and incubating for comparatively long periods. This avoided, to a large extent, the superimposing of leucocytes and carbon which could be seen in working with thick emulsions when smears were made immediately

upon mixing, without waiting for an incubation period. The following preparations were incubated for 45 minutes:

Mixtures	Carbon particles per 50 leucocytes Experiments I	II	III
A) Washed leucocytes + carbon emulsion + serum	411	215	75
B) Washed leucocytes + carbon emulsion + salt solution	76	53	10

These data show that the presence of serum was necessary for the phagocytosis of carbon particles. As to whether the serum is inactivated by heat for carbon particles, a dilute emulsion gave the following results:

Mixtures	Carbon particles per 50 leucocytes Experiments I	II
A) Washed leucocytes + carbon emulsion + serum	411	215
B) Washed leucocytes + carbon emulsion + serum, heated for 30 minutes at 60° C.	67	99

With a concentrated carbon emulsion the difference was less striking:

Mixtures	Carbon particles per 50 leucocytes
A) Washed leucocytes + carbon emulsion + serum	347
B) Washed leucocytes + carbon emulsion + serum, heated for 30 minutes at 60°C.	259

As regards the extent of inactivation of serum by heat for bacteria, apparently considerable variation occurs according to the concentrations employed.

In testing whether or not the heating experiment, as performed for the demonstration of opsonins, could be carried out with carbon particles, I obtained a uniformly negative result. An especially troublesome feature was the clumping of the carbon particles during the heating. I was, therefore, unable to repeat all of the opsonic experiments with an inert body such as the carbon particles employed. The experiments do show that the presence of serum does exert a favorable influence directly upon the leucocytes.

EFFECT OF THE SERUM UPON THE MOTILITY OF THE LEUCOCYTES.

As further evidence of the effect of serum upon leucocytes, it is found that when a drop of freshly drawn blood is collected in salt solution and examined upon a warm stage at 37° C., many of the leucocytes show definite amoeboid locomotion. In contrast to this, the leucocytes, when washed by centrifugalization for the preparation

of the emulsion in salt solution, show at most only an occasional pseudopod and are practically devoid of locomotion. Upon the addition of serum, careful examination of hanging drops upon the warm stage shows that they have regained both their intrinsic movements and to a limited extent their power of locomotion. In testing the motility in serum, heated for 30 minutes at 60° C., only a doubtful result was obtained. Sleeswijk[15] found that the leucocytes were non-motile when suspended with anthrax bacteria in salt solution and also practically non-motile when suspended with anthrax bacteria in heated serum. No observations were recorded for preparations with active serum.

CLUMPING ACTION OF THE SERUM.

The examination of opsonic preparations in hanging drops raises the question, Do the leucocytes, *in vitro*, move actively from place to place in search of bacteria, or do they merely engulf the neighboring organisms? Rosenow[28] found that after leucocytes have been killed by heat, the presence of opsonic serum causes the bacteria to be clumped around the leucocytes. Our results tend to support this conclusion.

An emulsion of leucocytes, after exposure to a temperature of 45° C. for 10 minutes, showed no motility whatever. (Rosenow has found that longer periods are necessary to destroy all phagocytic power.) Three ordinary opsonic preparations were then made with the heated emulsion, using (1) normal serum, (2) serum heated for 30 minutes at 60° C., and (3) salt solution. A hanging-drop mount was then made from each preparation. In the case of the normal serum, the majority of the staphylococci remained free, but a few were grouped about the leucocytes. In both the heated serum and the salt solution, the cocci showed no tendency to group about the leucocytes. At the end of 45 minutes, the hanging drops were allowed to dry, and after staining with Hasting's stain, the bacteria lying in contact with the leucocytes were counted.

Mixtures	Bacteria per 50 leucocytes Experiments I	 II
A) Heated leucocytes + staphylococcus emulsion + serum...........	296	244
B) Heated leucocytes + staphylococcus emulsion + heated serum....	27	60
C) Heated leucocytes + staphylococcus emulsion + salt solution.....	46	20

Although opsonins are held to be distinct from other immune

bodies, it is interesting, in considering this heterologous agglutination, that both high opsonic and high agglutinating serum is obtained in typhoid-immune animals[29, 30, 31] and also that in other diseases, such as staphylococcus infections, very low values are obtained for both agglutinins and opsonins.

ACTION OF THE SALTS IN PHAGOCYTOSIS.

For the demonstration of definite substances, in serum, essential for phagocytosis, but presumably without action on the bacteria, Dr. Cole suggested a study of the salts. The work of Bordet[32], and Joos[33], showing the necessity of salts for agglutination, indicates a similar possibility in phagocytosis.

In the preparation of salt-free phagocytic mixtures, the tonicity of the fluids was maintained with a 5.5 per cent solution of saccharose (Kahlbaum's). To prepare the leucocytic emulsion, 1 c.c. of blood was collected in 2 c.c. of saccharose solution containing 1 per cent of sodium citrate. This mixture of 3 c.c. was washed with 9 c.c. of isotonic saccharose solution and after collecting the leucocytes (about 0.3 c.c. volume), they were washed once more with 10 c.c. of saccharose solution.

In the preparation of the staphylococci and the serum, about 0.25 c.c. of staphylococcus emulsion in saccharose solution was digested with an equal volume of serum for 30 minutes at 36° C. The mixture was then diluted with 10 c.c. of saccharose solution, the bacteria were collected by centrifugalization, and an oese of the bacterial precipitate was re-emulsified in saccharose solution.

Mixtures	Bacteria per 50 leucocytes I
A) Washed leucocytes + sensitized bacteria + saccharose solution..........	47
B) Washed leucocytes + sensitized bacteria + salt solution................	258
C) Washed leucocytes + sensitized bacteria + serum.....................	291

In a repetition of this experiment, the staphylococci were emulsified in distilled water, digested with serum, and then washed with distilled water. An oese of the bacterial precipitate was emulsified as before, in saccharose solution.

Mixtures	Bacteria per 50 leucocytes II
A) Washed leucocytes + sensitized bacteria + saccharose solution..........	43
B) Washed leucocytes + sensitized bacteria + salt solution................	235

SUMMARY.

1. Any reaction, favoring phagocytosis, which may take place between *Staph. pyogenes aureus* and normal serum at 37° C., can be broken up by thorough washing.

2. Active phagocytosis of staphylococci is possible under conditions in which no opsonic action can be demonstrated, either by heating or by washing experiments.

3. One of the essential factors for phagocytosis, namely, the presence of salts, presumably does not owe its effect to an alteration of the bacteria.

4. Evidence of the action of the serum upon the leucocytes is shown by: (*a*) Their loss of motility in salt solution emulsions, and (*b*) The occurrence of phagocytosis of chemically inert bodies only in the presence of serum.

5. Further possibility of the mechanism of the action of the serum *in vitro* is seen in the clumping of the leucocytes and the bacteria.

Observers are not agreed as to what constitutes the proof of opsonic action. In the complex of substances present in the various bacteria and sera, one cannot expect that a reaction between them would never take place.

The experimental data at hand do not yet justify the conclusions for normal serum and staphylococci, a very typical case, that: (1) The predominating and essential factor in the conditions favoring phagocytosis is an alteration of the bacteria, nor that, (2) The serum by its presence is not of considerable importance in favoring the activity of the leucocytes.

At this opportunity, I wish to express my thanks to Dr. R. I. Cole, under whose direction this work has been carried on in the biological laboratory of the medical clinic.

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